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Generation and Evaluation of PEGylated Recombinant Human Acetylcholinesterase as an Optimal OP-Bioscavenger

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ABSTRACT:

We have shown in the past that recombinant human AChE (rHuAChE) can be converted into circulatory long-lived molecular forms by conjugation of polyethylene glycol (PEG) chains to lysine residues. In the present study, we determined the factors that govern circulatory residence of PEGylated AChE, and established the lysine mutant configurations that allow maximal circulatory longevity. We further demonstrated that PEGylated rHuAChEs are less immunogenic than non-modified enzyme. In addition, we incorporated favorable kinetic characteristics for OP-scavenging into the rHuAChE enzyme, utilizing genetic engineering methods. Thus, incorporation of the F338A mutation into circulatory long-lived PEG-hypolysine AChE resulted in an enzyme displaying reduced aging rates that could protect mice against repeated OP-compound exposures. Finally, we established a method for production of enzymatically active rHAChE at appreciable amounts and relatively low cost utilizing the *Pichia pastoris* yeast cell system.

Taken together, these different lines of study pave the way for large-scale production of PEGylated recombinant human AChE derivatives displaying catalytic and pharmacokinetic properties optimized for effective *in-vivo* bioscavenging of OP-compounds.

INTRODUCTION:

The primary role of acetylcholinesterase (AChE) is the termination of impulse transmission in cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. Some organophosphate (OP) compounds (*e.g.* the nerve agents sarin and soman) inhibit AChE irreversibly, and their acute toxicity is manifested in motor and respiratory failure following inhibition of AChE in the peripheral and central nervous system.

The high reactivity of ChEs towards OP-agents led to propose these biomolecules as exogenous scavengers for sequestration of toxic OP-agents before they reach their physiological target (Wolfe *et al*., 1987; Broomfield *et al*., 1991). AChEs react irreversibly and on a molar basis, with the OP agents and therefore, the amounts of AChE required for treatment are high. This limitation could be overcome provided that the OP-enzyme conjugates could be efficiently reactivated before the excess OP has reached its physiological target. This goal is difficult to attain especially in cases where the OP-AChE conjugates undergo catalytic post-inhibitory processes termed aging. In native

AChEs the spontaneous reactivation, through displacement of the phosphyl moiety from the active site, is usually very slow and unable to compete with the aging process, yet efficient enzyme reactivation can be achieved by various oxime nucleophiles. One of our goals was therefore to generate enzymes based on the human AChE (HuAChE) template, the OP-adducts of which are more readily reactivated and are resistant to aging, yet still retain their high reactivity towards the OP agents. To meet this challenge, we as well as others carried out studies designed for better understanding of the functional architecture of the AChE active center. These include X-ray crystallography (Sussman *et al.*, 1991; Harel, *et al.*, 1995; Kryger *et al.*, 1998, 2000; Millard *et al.*,1999); site directed mutagenesis and molecular modeling together with kinetic studies of the AChE muteins with substrates and reversible inhibitors (Ordentlich, *et al.,* 1993; 1996, 1998; Barak *et al.,* 1994; Shafferman *et al.,* 1992, 1995; Vellom, *et al.,* 1993; Radic *et al.,* 1993; Ariel *et al.,* 1998) . Indeed, based on these studies, we were able to generate novel enzymes that are more efficient in OP scavenging through the combined effects of improved activity toward the OP-agents together with higher resistance to the aging process (Ordentlich *et al.,* 1996; Shafferman *et al.,* 1996).

Exploitation of the bioscavenging potential of the recombinant bioengineered mutant derivatives of AChE depends on large-scale production systems. However, pharmacokinetic studies (Kronman, *et al.,* 1992; Mendelson *et al.,* 1998) have shown that recombinant enzymes generated by these systems, relying on either bacterial or mammalian cells, are retained in the circulation of experimental animals for much shorter periods of time than native fetal bovine serum AChE (FBS-AChE) or human serum butyrylcholinesterase (BChE). Extensive structural and biochemical analyses of varied forms of recombinant AChE, allowed us to determine an hierarchical pattern by which post-translation-related factors as well as specific amino-acid epitopes, determine the pharmacokinetic performance of the enzyme molecule in animal models (Kronman *et al*., 1995, 2000; Chitlaru *et al*., 1998, 2001, 2002). Optimization of some parameters affecting circulatory residence of rHuAChE through cellular and DNA engineering allowed us to generate a circulatory long-live recombinant HuAChE. In parallel, we demonstrated in the past that controlled conjugation of polyethylene glycol moieties to recombinant human acetylcholinesterase (rHuAChE), converts this enzyme into a circulatory long-lived species which resides for extended periods of time in the circulation of both mice and monkeys (Cohen *et al*., 2001, 2004). PEGylated AChE containing an average of 4 to 5 PEG moieties per enzyme, displayed maximal pharmacokinetic performance while retaining full catalytic activity, yet, examination of

the enzyme product revealed that it comprised a mixture of 3 major products, differing one from another in their PEG contents. Further attempts to increase the number of appended PEG units or to achieve uniform PEGylation under stringent conditions which favor highly effective PEG appendage, resulted in the generation of enzyme forms displaying severely reduced catalytic activity (Cohen *et al*., 2001).

Elimination of some lysine residues to prevent their appendage to PEG may be required to generate uniformly modified enzyme forms which retains full catalytic activity following PEGylation under stringent conditions which would otherwise reduce catalytic activity. However, elimination of selected lysine residues should be restricted to those that are not required for catalytic activity, do not play a role in the reactivity of the enzyme towards organophosphates, and are not crucial for maintaining the structural integrity of the enzyme.

The present report (see below, Accomplishments, Section I) summarizes the various lines of study in which the biological activity, pharmacokinetic performance and immunogenic properties of a large array of PEGylated lysine-modulated AChE forms were delineated. Based on these studies, we could determine the optimal human AChE enzyme configurations that could serve for the development of an effective bioscavenger against OP compound intoxication.

In many animal species BChE serves as the major circulating ChE, while AChE is present at considerably lower levels or not at all (Li *et al*., 2000), raising the question whether administration of large amounts of recombinant AChE or its derivatives may result in altered disposition of the enzyme in various tissues. Furthermore, the presence of polyethylene glycol tails on ChEs may enhance their accumulation in vital organs and thus may exert some negative biological effects. To address these issues, we monitored the tissue distribution of PEGylated rHuAChE in mice and Guinea pigs as detailed below (Accomplishments, Section II). In addition we examined the welfare of the animals following administration of PEGylated rHuAChE, and our results are presented in this section as well.

The end product, which is to serve as the optimal HuAChE-based bioscavenger, should, in addition to its high circulatory longevity, comprise favorable kinetic traits. To this end, various properties, determined through multifaceted studies of AChE carried out over the years (see Shafferman *et al*., 2005 and references within), should be incorporated into the design of the optimal bioscavenger. For optimal reactivity of the HuAChE-based bioscavenger, the enzymatic component of the AChE+oxime reactivator scavenging

system should be characterized by high reactivity toward OP agents and long-term reactivability of the corresponding OP-enzyme adduct. In other words, the enzyme should remove most of the OP agent from the circulation before significant distribution to the target tissues takes place. In addition, the resulting OP-adducts, ideally, should age slowly compared to the rate of enzyme reactivation by the oxime component of the scavenging system. In case of the AChE-soman adduct this is a difficult goal to achieve since mutations that affect aging also retard the phosphylation process. The only exception we found to this pattern is the F338A HuAChE which exhibits near to wild type like reactivity toward soman and yet the resulting adduct ages approximately 100-fold more slowly (Ordentlich *et al*., 1999). Thus, the F338A mutation appears to confer favorable biochemical traits, which should be considered for incorporation into the PEG-AChE bioscavenger platform.

In the present study (see Accomplishments, Section III) we examined the ability of PEGylated rHuAChE to serve as an efficient prophylactic agent against OP compound intoxication in mice. In addition, we prove that incorporation of the F338A mutation into the PEG-rHuAChE mold, indeed confers the enzyme with the ability to effectively protect mice against repeated OP exposures.

Large quantities of cholinesterase are required for the utilization of these enzymes as stoichiometric bioscavengers of OP-compounds. In recent years, the methylotrophic yeast *Pichia pastoris* cell system has been implemented for large-scale production of heterologous proteins. The advantages of this particular expression system, include: (a) exogenous proteins produced at high levels in *Pichia pastoris* can be directed to the extracellular medium (b) the capability of performing many eukaryotic posttranslational modifications, including disulfide bond formation and proteolytic processing, and (c) the ability to rapidly grow on inexpensive media to high cell densities.

Comparison between the human AChE gene and the genome of *P. pastoris* demonstrates that the coding sequence of the HuAChE gene is characterized by a significantly higher GC content. Codon usage in the HuAChE gene also differed significantly from that of *P. pastoris.* To allow optimal expression of human AChE in *P. pastoris,* we designed and generated a synthetic HuAChE gene (sAChE) of lower GC content that codes for authentic human AChE, utilizing nucleotide codons which will be compatible with efficient expression in *P. pastoris*. During the course of this project, studies aimed to generate a *P. pastoris* cell clone that expresses the synthetic gene and secretes active recombinant HuAChE at high levels, were carried out at our laboratory. This was

followed by the establishment of a fermentation procedure that allowed production of large amounts of enzyme at bench-scale, which was then subjected to extensive biochemical and pharmacokinetic characterization. These results are summarized in the present report (Accomplishments, Section IV).

ACCOMPLISHMENTS:

I. Determination of human AChE enzyme configurations exhibiting optimized pharmacokinetic and immunogenic properties following PEGylation

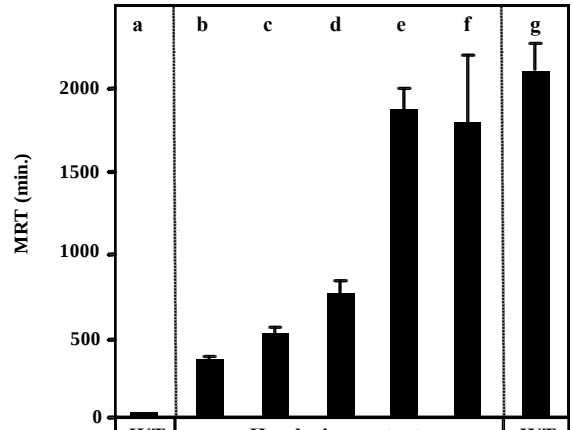
(1) As a first step towards the evaluation of the effect of lower lysine contents on AChE pharmacokinetics, we generated a series of different hypolysine AChE expression vectors which code for C-terminal truncated human AChE in which one or two lysine residues are replaced by alanine. These constructs were introduced into HEK-293 cells, and stable pools of each of the different AChE mutants were generated. In all cases, the specific activity of the hypolysine mutants did not deviate in a significant manner from that of the wild type enzyme (6.5 U/μg), indicating that the removal of any of the different pairs of lysine residues did not alter the kinetic performance of the enzyme.

(2) Based on these findings we generated different multilysine AChE mutants in which three or more lysine residues are replaced by alanine. These plasmid vectors were introduced into cells of the HEK-293 lineage and secreted AChE activity was determined in the conditioned media 48 hours post-transfection. Secreted enzymatic activity could be determined for all AChE hypolysine mutants. To evaluate the structural and functional integrity of the recombinant enzymes, the amounts of secreted protein of the various hypolysine AChEs were determined by capture ELISA, using polyclonal anti-human AChE antibodies of mouse and rabbit source. In all cases, the enzymatic activity to protein mass ratios (specific activity) were similar, differing from that of the wild type rHuAChE by no more than a factor of 3, indicating that the elimination of selected lysine residues did not cause any gross alteration in the enzyme architecture. Thermal stability at 37^0C of all of the mutated AChE forms was also similar and differed from that of the wild-type enzyme by no more than a factor of ~2. Since all of the selected AChE mutants display unaltered catalytic and structural performance, all of the transfectants were subjected to a selection process, to give rise to stable cell pools expressing the enzymes in a constitutive manner.

(3) Cell clones stably expressing high levels of the various hypolysine AChEs were generated and measurement of secreted AChE activity revealed that production levels of all the various AChE derivatives were within the range of 5-20 units per 10^6 cells/24 hours, and thus were similar to that of wild type enzyme. All of the stable mutated-AChE expressor cells were propagated at large-scale, and secreted AChEs were purified. The purified hypolysine mutated AChEs were subjected to PEGylation under conditions that favor PEG saturation. The fully PEGylated hypolysine AChEs, which carry 1 to 5 PEG chains per enzyme subunit in accordance with their available PEG target sites, exhibited undiminished catalytic activity following full PEGylation. The finding that all of the hypolysine mutated AChEs display unaltered catalytic activity upon PEGylation, demonstrates that reduction of lysine PEG-target sites can provide the means to generate uniformly PEGylated AChE exhibiting undiminished catalytic performance.

(4) To determine the effect of PEG number on the circulatory residence of AChE, uniformly PEGylated AChE carrying a single PEG chain (PEG-K23A/K53A/K332A /K348A/K470A/K496A/K538A); 2 PEG chains (PEG-K23A/K53A/K332A/K348A /K496A/K538A); 3 PEG chains (PEG-K23A/K332A/K348A/K470A/K496A); 4 PEG chains (PEG-K23A/K348A/K470A/K496A) and 5 PEG chains (PEG-K23A/K332A /K348A) were administered to mice. Elimination of the various fully PEGylated hypolysine AChEs was monitored by determining residual AChE activity in blood samples removed at various time points, and Mean Residence Time (MRT) values were calculated (Figure 1). In general, enzyme forms exhibiting an increase in appended PEG chains were retained in the circulation for longer periods of time. The appendage of a single PEG chain to the alysine AChE (where PEG can be appended to the terminal primary amine of the enzyme only), significantly increased the MRT of the molecule from 42 minutes to 365 minutes. Appendage of additional PEG chains was accompanied by a gradual increase in circulatory residence, so that fully PEGylated monolysine, dilysine and trilysine AChEs displayed MRT values of 520, 770 and 1860 minutes, respectively. Appendage of a fifth PEG moiety to AChE (by PEGylation of the tetralysine-AChE) did not contribute significantly to the circulatory residence of the PEGylated enzyme. The MRT values of the enzyme forms carrying 4 and 5 PEG chains was within the range of 1800-1900 minutes, comparable to that of the wild type enzyme randomly PEGylated under conditions which favor the appendage of 4-5 PEG moieties (MRT ~ 2000±200 minutes). These results clearly demonstrate that the number of appended PEG chains plays a pivotal role in determining the circulatory residence time of

the enzyme and that the rank order of circulatory longevity of these molecules was dependent on the number of PEG appendages up to a certain threshold: 5=4>3>2>1>0.

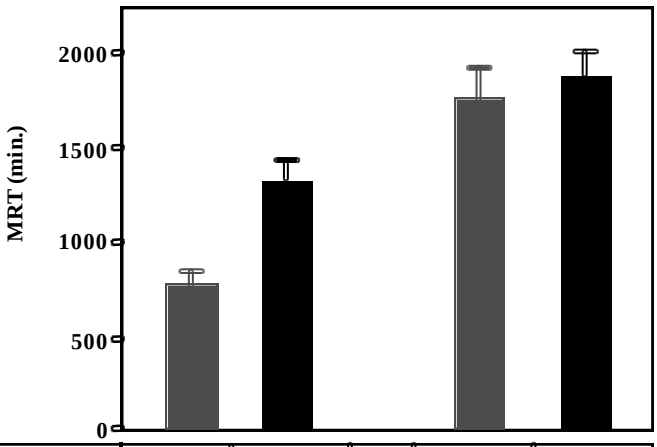


	WT	Hypolysine mutants					WT
PEG No.	0	1	2	3	4	5	4-5
PEG sites	-	N-ter	N-ter 470	N-ter 53 538	N-ter 53 332 538	N-ter 53 470 496 538	Hetro-genous
MRT (min)	42 ±8	365 ±20	520 ±30	770 ±70	1860 ±140	1805 ±270	2000 ±200

Figure 1: Mean Residence Time (MRT) values of PEGylated wild-type and Lys-Ala hypolysine AChEs. Bars indicate the MRT values (minutes) ± standard deviation of the different enzyme forms. The number and sites of available sites for PEGylation are indicated for each enzyme form (N-ter denotes the presence of a PEG chain at the N-terminus of the enzyme molecule). Lane a: non-PEGylated wild type AChE, lane g: wild-type AChE that was heterogeneously PEGylated under conditions favoring the appendage of an average of 4 to 5 PEGS per enzyme subunit.

(5) To examine whether the actual locations of the PEG chains also plays a role in determining the circulatory of the PEGylated AChE, we monitored the pharmacokinetic performance of hypolysine mutants, which allow appendage of equal numbers of PEG chains at different locations at the surface of the enzyme (Figure 2). To this end, we examined the pharmacokinetic behavior of an additional dilysine and trilysine AChE (K470/K538 AChE and K53/K348/K470 AChE, respectively), which were chosen based on the spatial location of their available PEG appendage sites within the 3D-model of human AChE. Indeed, PEG-K470/K538 AChE was retained in the circulation for substantially longer periods of time than its PEGylated dilysine counterpart, K53/K538 AChE (MRT values of 1320 minutes and 770 minutes, respectively). Likewise, PEG-K53/K348/K470 AChE, resided in the circulation of mice for a shorter period of time

than its PEGylated trilysine counterpart, K53/K332/K538 AChE (1740 and 1860 minutes, respectively). Thus, pairs of AChE forms containing the same number of appended PEGs and thereby exhibiting identical molecular weights, may nevertheless reside in the circulation for different spans of time due to the unequal spatial distribution of their PEG chains. In conclusion, the actual location of the appended PEG chains also plays an important role in determining the pharmacokinetic behavior of chemically modified enzyme.



PEG No.	3	3		4	4
PEG sites	N-ter 53 538	N-ter 470 538		N-ter 53 348 470	N-ter 53 332 538
MRT (min)	770 ± 70	1320 ± 120		1740 ± 210	1860 ± 140

Figure 2: Mean Residence Time (MRT) values of PEGylated wild-type and Lys-Ala hypolysine AChEs. Comparison of MRT values of 2 different dilysine and 2 different trilysine AChEs. Mean Residence Time (MRT) values of PEGylated wild-type and Lys-Ala hypolysine AChEs. A. MRT values of 5 different hypolysine AChEs containing 1 to 5 appended PEG chains. Bars indicate the MRT values (minutes) ± standard deviation of the different enzyme forms. The number of available sites for PEGylation, as well as the sites for PEGylation are indicated for each enzyme form (N-ter denotes the presence of a PEG chain at the N-terminus of the enzyme molecule).

(6) In addition to their pharmacokinetic properties, different PEGylated AChEs should be examined for their immunogenic traits in view of the possibility that some of these forms may be more immunogenic than others, due to either a reduced PEG load, or to particular spatial distributions of the PEG residues which results in the generation of exposed surface regions that are accessible to the immune system. To this end, seven different human AChE derivatives- alysine AChE, K470 AChE, K470/K538 AChE, K53/K538 AChE, K53/K348/K470 AChE, K53/K332/K538 AChE, and K53/K470/K496/K538 AChE were produced at large-scale, and milligram amounts of the various AChEs were purified. The various AChEs were then PEGylated under conditions that promote full saturation without compromising catalytic activity. SDS-PAGE analysis allowed us to determine that each of the PEGylated products appears as a single uniform band, and that the various PEG AChEs carry 1 to 5 PEG chains, in accordance with their available PEG target sites. All of the PEGylated AChEs, as well as randomly PEGylated wild type AChE and non-PEGylated wild-type and alysine AChEs were subcutaneously administered to mice (10 μg/mouse; 10 mice/AChE form). Mice were re-injected with the various PEG AChEs at monthly intervals, and screened for the presence of anti-AChE antibodies (Table 1). Following the second injection, anti-AChE antibody titers in sera sampled from mice administered with PEG-AChEs carrying 3 to 5 PEG moieties per enzyme, were with the range of 100 to 300, while sera from mice that were administered with PEG-AChEs with 0 to 2 PEG chains displayed titer values within the range of 1500 to 2200. This difference in anti-AChE titers after injection with low-level PEGylated and high-level PEGylated AChEs was even more conspicuous following the third injection (Table 1). Anti-AChE antibody titers in all sera from mice administered with AChEs carrying 3-5 PEG chains were within the narrow range of 1000-1600, while titers in sera from mice administered with AChEs carrying 0-2 PEG chains were ~10-fold higher, within the range of 12,000-13,500. Thus, appendage of 3 PEG chains to AChE is both required and sufficient for significant reduction of the immunogenicity of the enzyme molecule even in heterologous systems (human enzyme in mice). The appendage of additional PEG chains does not result in further reduction in immunogenicity, suggesting that 3 PEG chains of 20,000 Da provide the AChE molecule with an adequate coating and effectively screen the enzyme from the host immune system.

	α-AChE Antibody titers^{-1}		
Species	**No. of PEGs**	**Post 2nd injection**	**Post 3rd injection**
WT	0	1500 (1.9)	12800 (2. 3)
WT	4-5	290 (3.7)	1050 (3.6)
K53/K470/K496/K538	5	180 (3.9)	1500 (4.1)
K53/K332/K538	4	140 (2.7)	1600 (3.7)
K53/K348/K470	4	130 (2.7)	1200 (2.5)
K53/K538	3	280 (4.9)	1600 (4.5)
K470/K538	3	110 (2.9)	1400 (4.6)
K470	2	1600 (2. 0)	12800 (1. 8)
alysine	1	2200 (2. 9)	13500 (1. 7)
alysine	0	1500 (2. 3)	12000 (1. 8)

Table 1: Anti-AChE antibody titers in sera of mice repeatedly administered with various non-PEGylated and PEGylated recombinant human AChEs. Mice (10 mice/group) were administered at monthly intervals with 10 μg/mouse of either non-PEGylated or PEGylated WT or hypolysine rHuAChEs. Anti-AChE antibody titers were determined 3 weeks following the 2nd and 3rd injection by ELISA using highly purified rHuAChE as the capture antigen. Titers are presented as geometric mean values with standard deviations within parentheses.

(7) Taken together, these studies demonstrate that fully PEGylated K23A/K348A/K470A/K496A trilysine AChE, and K23A/K332A/K348A tetralysine AChEs (which contain 4 and 5 PEG chains/enzyme subunit, respectively), exhibit MRT values of 1860±140 and 1800±170 minutes, respectively, and therefore seem to be the optimal enzyme configurations for the biological scavenging of OP compounds, in terms of their circulatory longevity. The finding that these enzyme forms exhibit also reduced immunogenicity, underscores their potential to serve as therapeutic bioscavengers of organophosphate compounds.

II. Determination of the effect of PEG-AChE administration on animal physiology

(1) To evaluate the feasibility of intramuscular pretreatment with PEGylated rHuAChE, we monitored the pharmacokinetic performance of *i.m.* administered PEGylated rHuAChE in mice (Figure 3). The rate of elimination from the serum of the PEGylated rHuAChE was very similar to that exhibited for this enzyme form following intravenous administration and the bioavailability of the PEG-modified AChE was calculated to be 56%. In comparison, serum HuBChE and FBS-AChE displayed bioavailability values of 54% and 29%, respectively, following intramuscular administration to mice. Thus, PEG-conjugation does not seem to deleteriously affect the ability of the enzyme to be directed to the circulation.

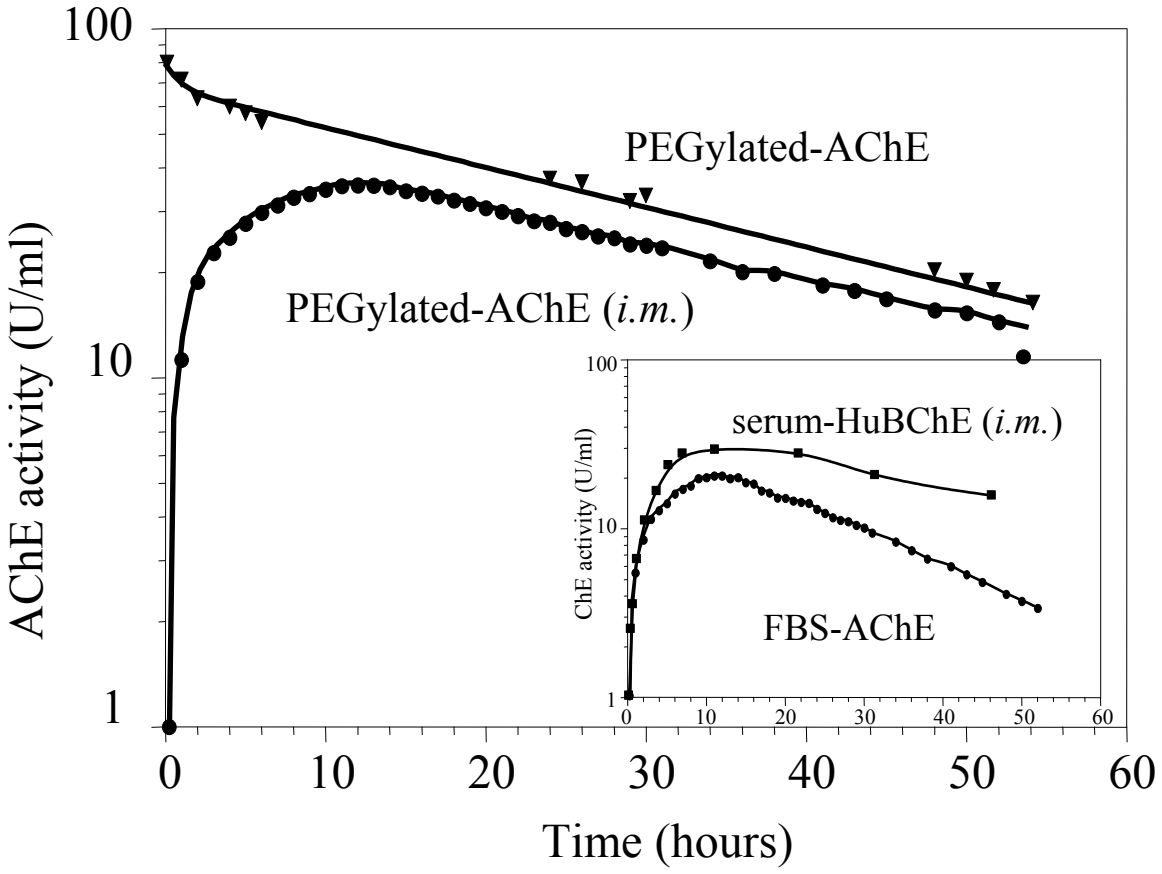


Figure 3: Circulatory clearance profiles of PEGylated-rHuAChE administered by different routes. PEGylated AChE (200U) was administered either *i.v.* (triangles) or *i.m.* (circles) to 3 mice. Blood samples withdrawn at various time points were assayed for AChE activity and values were corrected for background hydrolytic activity in the blood. Values are presented as AChE units per ml blood. Bioavailability of *i.m.* administered PEGylated rHuAChE was calculated as $AUC_{i.m.}/AUC_{i.v.}$ **Inset:** Clearance profiles of FBS-AChE and serum human BChE administered *i.m.* Bioavailability values of these two enzymes were calculated by comparing these clearance profiles to those obtained following *i.v.* administration of the corresponding enzyme forms (Raveh *et al.*, 1993; Kronman *et al.*, 2000).

Further experiments, carried out in Guinea pigs, demonstrated that *i.m.* administered PEGylated rHuAChE resides in the circulation for extended periods of time in this animal model as well. Taken together, the extended circulatory residence times of PEGylated rHuAChE in both mice and Guinea pigs suggests that extravascular pretreatment with PEG-rHuAChEs may serve as an effective mode for protection against OP toxicity.

(2) Comparative biodistribution analysis allowed to determine that recombinant human AChE enzyme intravenously administered to mice, did not accumulate in any of the organs examined, whether or not the enzyme was chemically modified by PEG-conjugation. In fact, PEGylated rHuAChE enzyme levels in all organs was very low at all time points and did not exceed 2% of plasma levels. However, tissue distribution might be affected by the route of administration of the protein and therefore, we monitored PEG-AChE biodistribution, following intramuscular administration, as well (Figure 4).

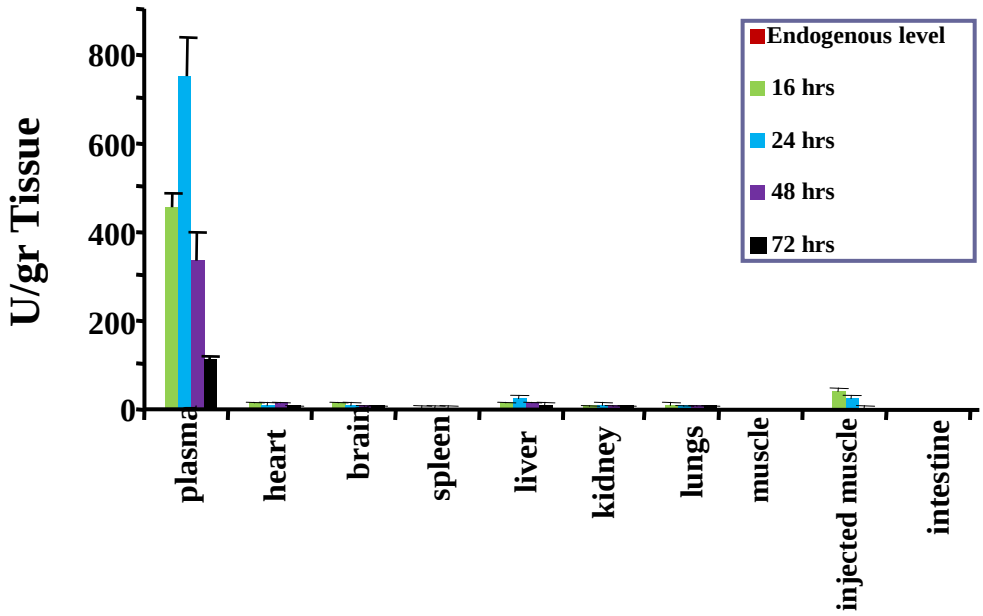


Figure 4: Tissue distribution of PEGylated-rHuAChE. PEGylated-rHuAChE was administered (3000 U) *i.m.* to each of 3 mice and at each time point, blood samples and tissues were removed and AChE activity was measured in tissue homogenates.

With the exception of the muscle into which the enzyme was injected, enzyme levels in all organs was less than 2% of plasma levels at all time points, and most likely represent residual blood in these organs. In the injected muscle, enzyme levels at 16 hours post-administration were approximately 10% of plasma levels, while at 24 and 48 hours post-

administration, enzyme levels in the injected muscle were 2.5% of plasma levels and near to background levels, respectively. Thus, i.m. administered PEG-AChE was eliminated quite efficiently from the site of injection and did not accumulate in any of the major organs.

(3) To determine whether extravascular administration of PEG-AChE affected the welfare of experimental animals, groups of mice were administered PEG-AChE (2 nmole/mouse) intramuscularly and then monitored at various time points for body weight and temperature, blood glucose levels, blood biochemistry and hematology. No reduction in body weight was detected following enzyme administration, and from day 4 onward, body weight increased in a manner similar to that of control mice that were administered PBS only. Likewise, blood glucose levels of the PEG-AChE administered mice at both 48 hrs and 7 days post-administration were similar to those measured in control mice, and were well within the normal value range. All other blood chemistry values examined in the PEG-AChE administered mice were also within the normal value range and did not differ in any significant manner from the corresponding values displayed in control mice. PEG-AChE administered mice also displayed normal differential blood counts that were essentially indistinguishable from those of control mice, both at 48 hrs and 7 days post-enzyme administration. Similar results were obtained in Guinea pigs. Thus, extravascular administration of PEGylated HuAChE did not compromise animal physiology or blood chemistry of mice or Guinea pigs in any detectable manner. Taken along with the finding that PEGylated HuAChE does not accumulate in any organ tested, we conclude that intramuscularly administered PEG-AChE can serve as a safe means for therapeutic treatment of OP compound poisoning without exerting any deleterious effect on the treated subjects.

III. Generation of selected human AChE derivatives with improved bioscavenging properties and optimal pharmacokinetic performance

(1) To examine the bioscavenging performance of PEGylated AChE, mice were administered 1.2 nmole PEGylated AChE and then subjected to a soman challenge of 2.5 LD_{50}. As in the case of the non-modified AChE, all of the mice pretreated with the PEGylated version of the enzyme survived challenge and exhibited no more than mild symptoms of intoxication. Similarly, AChE and PEGylated AChE protected mice against sarin and VX, in an equal manner. Taken together, these results clearly demonstrate that the appendage of PEG moieties to rHuAChE did not compromise the ability of the enzyme to effectively interact and neutralize various OP compounds, so that PEGylated AChE can fully protect mice against soman, sarin or VX intoxication.

(2) To examine the prophylactic potential of PEG-modified AChE when administered a long time before the exposure to CW agents, 3.2 nmole/mouse of PEGylated rHuAChE, was administered intravenously to mice, and 22 hours later the mice were exposed to 1.5 LD_{50} VX. All of the mice survived exposure. In contrast, mice pretreated with the same amounts of circulatory short-lived non-PEGylated rHuAChE died within minutes of the challenge. Thus, recombinant AChE in its PEGylated version confers protection to mice against OP compounds even many hours after administration of the enzyme. To further assess the ability of the PEGylated enzyme to provide protection against multiple exposures to OPs, these mice were subjected 2 hours later to a second challenge of 0.9 LD_{50} VX. Following re-exposure all the PEGylated rHuAChE pretreated mice survived. Overall, during this experiment a total dose equal to 2.4 LD_{50} of VX was administered to mice, and under these conditions, PEGylated rHuAChE conferred greater protection (100% survival) than HuBChE (25% survival).

(3) The high bioavailability determined for PEGylated rHuAChE (Section II, above) suggests that extravascular pretreatment with PEGylated rHuAChE a long time before the exposure to CW agents will also effectively protect against OP toxicity. To further examine this issue, mice were intramuscularly administered with 3 nmol of either serum HuBChE or PEGylated rHuAChE, and after 20 hours were challenged intravenously with 1.3 LD_{50} VX. Nearly 90% of mice pretreated with PEGylated rHuAChE survived challenge and exhibited only mild symptoms of toxicity. In contrast, only 60% of the serum HuBChE-pretreated mice survived, while exhibiting severe signs of toxicity.

(4) In a series of experiments, we measured the rate of hydrolysis, inhibition kinetics, phosphylation rates, stereoselectivity and rates of reactivation of OP-adducts, and

determined that neither the introduction of the F338A mutation nor the chemical modification of the enzyme by PEG conjugation, deleteriously affected the bioscavenging properties of the enzyme. The only discernable difference between F338A-AChE and WT-AChE was their differential rate of aging. Controlled PEG conjugation of F338A-AChE did not affect its rate of aging, so that both the non-PEGylated and PEGylated versions of F338A-AChE are characterized by significantly lower rates of aging than WT-AChE. This low rate of aging may provide the enzyme with an advantageous trait in terms of its ability to serve for prophylactic treatment of OP intoxication.

(5) To demonstrate that the slow aging rate of the soman-inhibited PEG F338A-AChE allows restoration of its activity in the bloodstream by oxime reactivation while soman-inhibited WT AChE remains refractive to this treatment, mice were administered either with PEG-WT-AChE or PEG-F338A-AChE (4 nmol/mouse) and 1 minute later exposed to the highest dose of soman (6.5 nmole/mouse, 5.4 LD_{50}) that is compatible with complete survival of the enzyme-administered mice. Measurement of AChE activity in blood samples removed 10 minutes later allowed us to determine that 70% of both enzyme forms were inactivated. HI-6 (50mg/kg) was then administered *i.v.* to the mice and enzyme reactivation was monitored in blood samples removed at various timepoints. In the case of the WT-AChE administered mice, the enzyme remained inhibited throughout the experiment, while in the case of the F338A-AChE-administered mice, the level of circulating active enzyme increased by 67% within 10 minutes following HI-6 administration (Figure 5A).

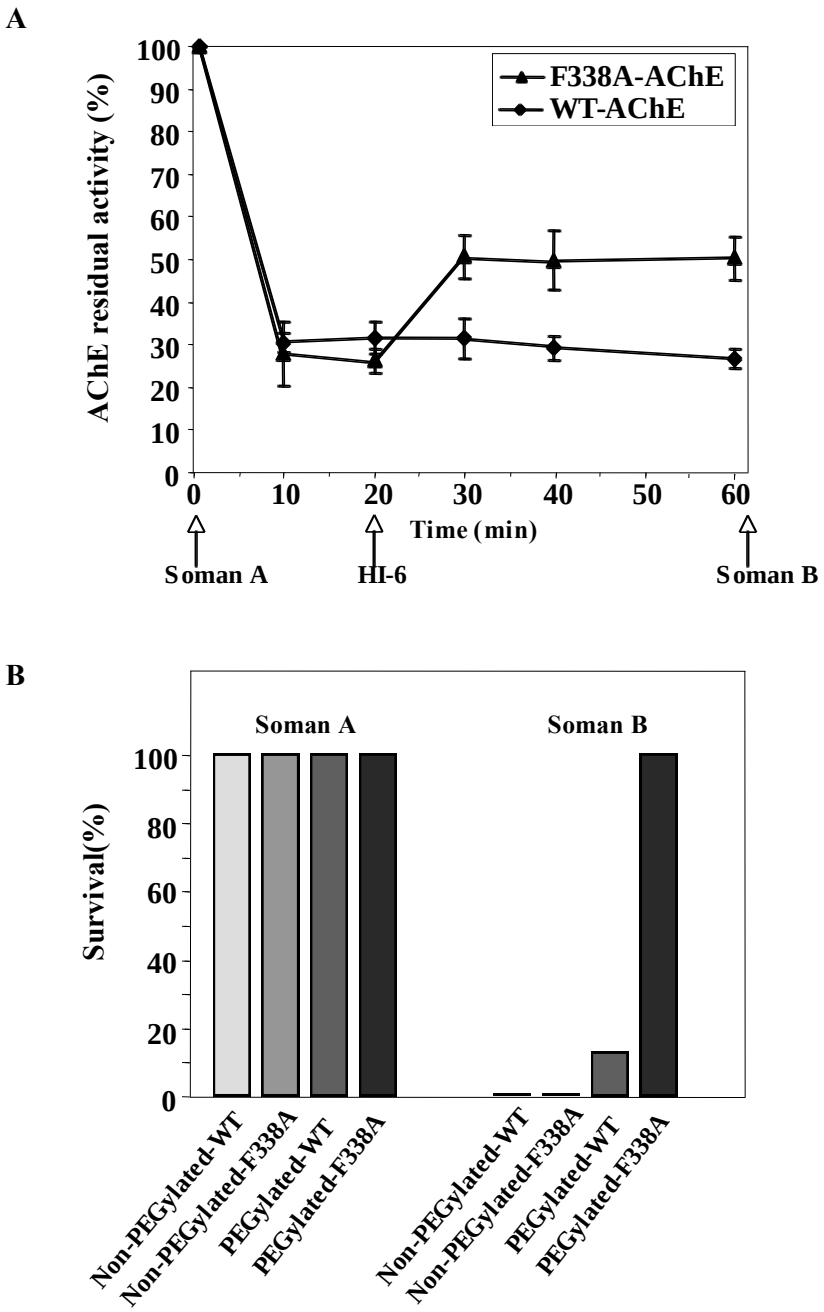


Figure 5: *In-vivo* reactivation and protective potential against repeated soman exposure of WT- and F338A-AChEs. Mice pretreated with non-PEGylated or PEGylated WT- and F338A- AChEs (2 nmol/mouse, i.v., T=0) were exposed to soman (4 nmol/mouse, i.v., T=1 min.), treated with HI-6 (50 mg/kg, iv, T=21 min.) and re-exposed to soman (4nmol/mouse, i.v., T=61 min.). *A.* AChE activity was monitored in blood samples removed at various timepoints from mice pretreated with the PEGylated AChEs. Activity is expressed as percent of input values, determined immediately after administration of the exogenous PEG-AChEs. Times of AChE administration, first soman exposure (1st soman), HI-6 treatment and second soman exposure (2nd soman) are indicated by arrows. *B.* Percent survival following the first and second soman exposures of mice (n=6) pretreated with the different AChE enzyme forms.

(6) To assess the ability of PEG-F338A-AChE to confer protection against repeated soman exposure, mice were administered PEGylated and non-PEGylated versions of both WT-AChE and F338A-AChE and then exposed to 5.4LD_{50} of soman. The mice, all of which survived this soman challenge and displayed no more than mild symptoms of OP-intoxication, were administered HI-6 twenty minutes later and after an additional 40 minutes, were re-exposed to a second soman challenge of 4LD_{50}. As expected, mice pretreated with the circulatory short-lived non-PEGylated versions of AChE succumbed to the second soman challenge, regardless of whether the WT or F338A version of the enzyme was administered (Figure 5B). PEGylated WT-AChE protected only 16% of the mice, even though this enzyme form exhibits long-term circulatory residence, so that at the time of the second soman challenge, 80% of the input enzyme still resides in the circulation. In contrast, 100% of the PEG-F338A-AChE pretreated mice survived the second soman challenge (Figure 5B). Since the pharmacokinetics of PEG-F338A-AChE and PEG-WT-AChE are very similar, the marked difference between the survival rates of the mice administered with these two enzyme forms necessarily stems from the differential ability of these enzymes to undergo reactivation by *in-vivo* oxime treatment. Based on these findings, it seems indeed that the *in-vivo* reactivation of soman-inhibited PEG-F338A AChE can be harnessed to provide effective protection against multiple exposures to OP-compounds.

IV. Evaluation of the possibility for large-scale cost-effective production of human AChE in a *Pichia pastoris*-based yeast expression system

(1) *Pichia*- expression vectors containing the synthetic human AChE coding regions were introduced into the KM71H (Mut^S phenotype), and GS115 (Mut^+ phenotype) *Pichia* strains. Screening for high AChE producer cells was carried out in a multi-well plate format (Figure 6). In total, 100 to 120 individual clones of each cell type were examined for secreted AChE activity and cell density. Five different KM71H-HuAChE and GS115-HuAChE cell clones exhibiting the highest levels of secreted AChE in the multi-well screening procedure, were further propagated in shake flasks and re-examined for AChE production. Secreted AChE levels of these high-level producer clones were within 2-4 U/ml/96hrs. A single clone, KM71H-HuAChE/#646, exhibiting the highest secreted AChE level, was selected for further studies.

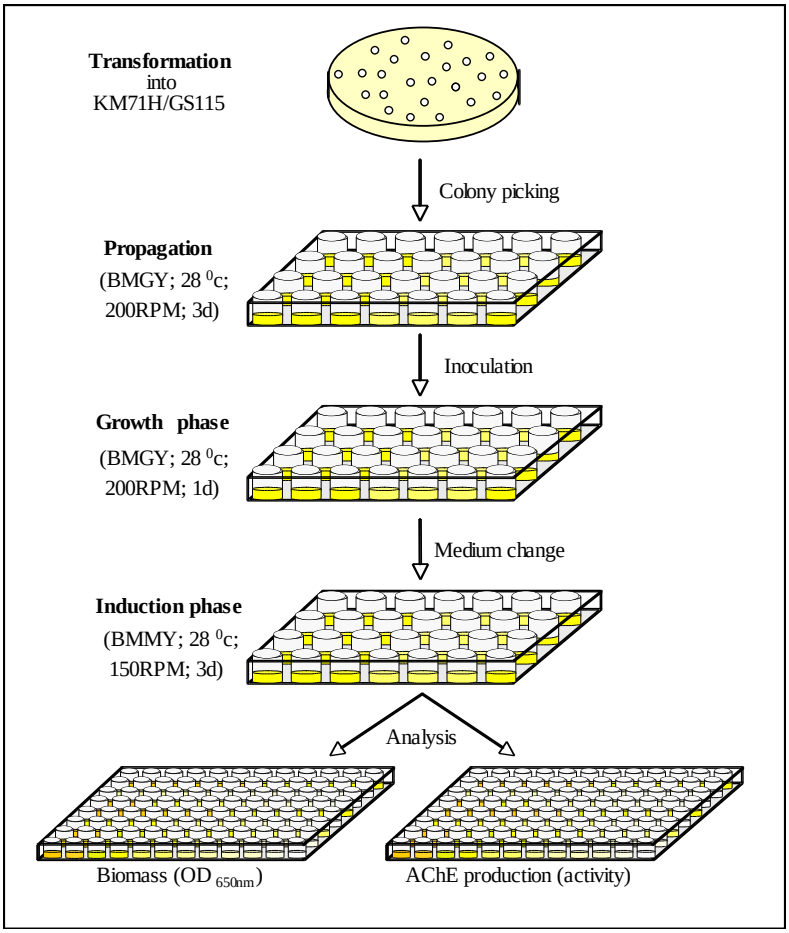


Figure 6: Schematic presentation of the screening procedure for *Pichia pastoris* transformant cells expressing and secreting high-levels of AChE.

(2) We utilized the KM71H-HuAChE/#646 AChE expressor clone for the establishment of a protocol for large-scale production of human AChE in the *Pichia pastoris* expression system. Fermentations were carried out at 30ºC in a BioFlo 2000 fermentation system (New Brunswick Scientific Company, Edison, NJ) having a 4 L working volume, with the dissolved oxygen control set at 30% oxygen saturation. Growth medium was based on BMGY medium (Invitrogen), which was modified to include magnesium and larger amounts of yeast extract and peptone. The basic fermentation protocol consisted of 3 stages: (a) biomass accumulation up to 10-20% WCW at pH 5.8, (b) pH adjustment to pH 7 for two hrs under glycerol limitation, and (c) 0.3-0.4% MeOH induction for at least 72 hrs by continuously feeding a 100% (v/v) MeOH solution at a slow rate, controlled by a MeOH sensor and controller that maintained the methanol concentration at a constant set point.

(3) Meticulous studies allowed us to determine that glycerol spiking improves both the continuous growth of cell mass and accumulated AChE levels over an extended period of time. By optimizing glycerol levels within the course of the fermentation by controlled spiking at predetermined intervals, we were able to bring about a >100-fold increase in

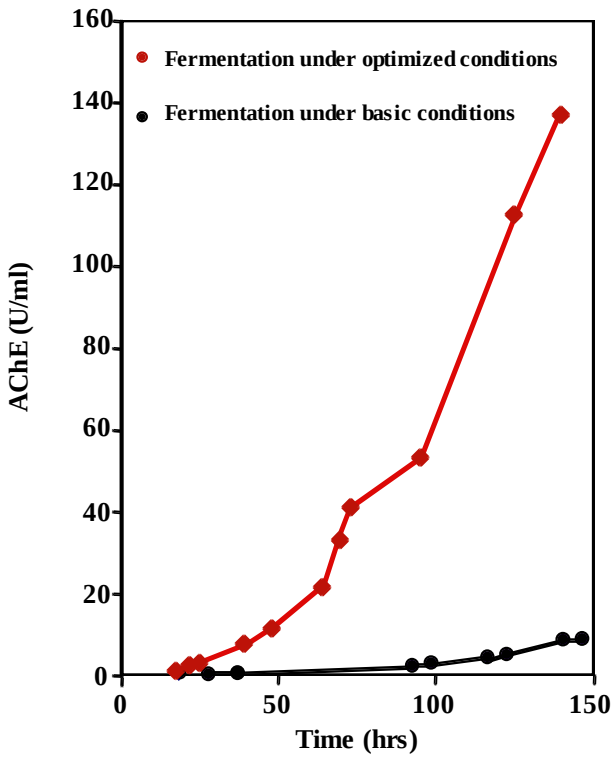


Figure 7: Comparison of AChE (U/ml) accumulation in the culture medium during fermentation runs under basic and optimized conditions. AChE activity was determined in samples collected at different time points during the induction phase of fermentations. Optimized conditions include glycerol spiking (see Text).

the yield of secreted AChE, as compared to initial fermentations (Figure 7). Moreover, under the optimized set of conditions, this enzyme yield was reached at shorter fermentation time periods than before. Thus, following 130 hours of induction, secreted AChE accumulated to 140 U/ml.

(4) We carried out a series of experiments to determine the conditions for purification of *P. pastoris*-produced rHuAChE. We found that microfiltration and ultrafiltration of the *P. pastoris* growth medium prior to loading on procainamide columns improved binding so that ~70% of the enzyme product could bind to the column and ~60% of the enzyme could then be eluted. The purified *P. pastoris*-produced rHuAChE appears on SDS-PAGE as a band doublet, which upon deglycosylation resolves into a single faster-migrating band. Thus, the *P. pastoris*-produced rHuAChE apparently consists of differently glycosylated products, which upon glycan removal, converge into a single product.

(5) We demonstrated that the *P. pastoris* enzyme product can undergo PEGylation as efficiently as the HEK-293-produced rHuAChE, and that the high-mannose yeast-related glycans of the *P. pastoris* enzyme product do not interfere with rHuAChE PEGylation. *Pichia pastoris*-derived rHuAChE was produced at large amounts, the enzyme product was purified to homogeneity, subjected to PEGylation and then examined for its pharmacokinetic performance (Figure 8). The PEGylated version of *Pichia pastoris*-derived rHuAChE resided in the circulation of mice for extended periods of time, displaying an MRT value of 1240 minutes. Thus, PEGylation of the *Pichia*-produced enzyme resulted in a greater than 400-fold increase in circulatory longevity of the recombinant enzyme.

(6) Taken together, we have established a method for production of enzymatically active recombinant HuAChE at appreciable amounts and relatively low cost utilizing a eukaryotic microorganism-based expression system. These studies, which were carried out at bench-scale, can serve as a guideline for the establishment of a large-scale cost effective production system, for the generation of the large amounts of rHuAChE required for stoichiometric bioscavenging of OP compounds.

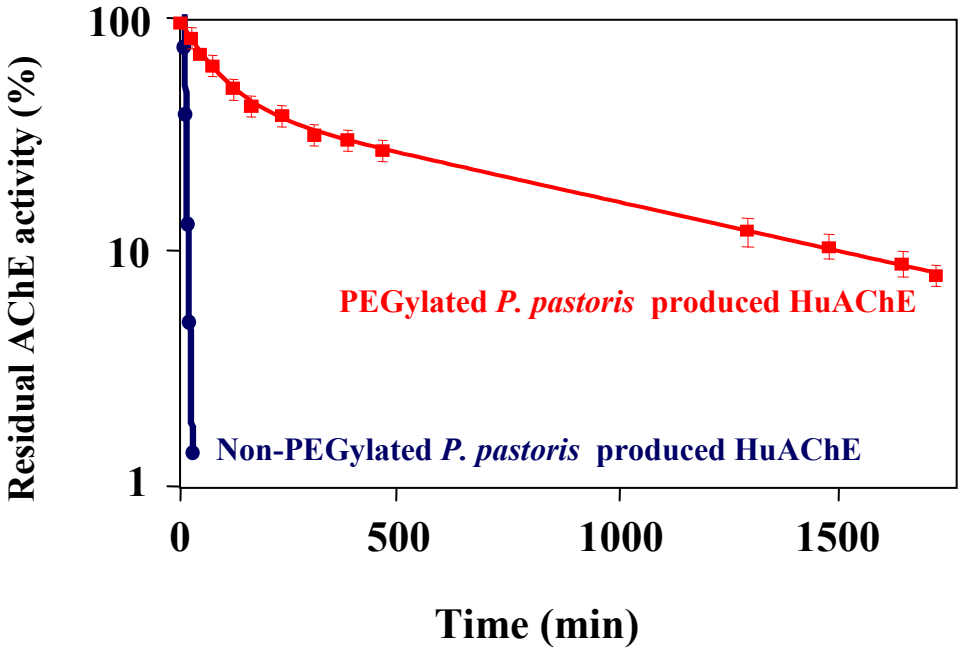


Figure 8: Pharmacokinetic profile of *Pichia pastoris*-produced rHuAChE before and after PEGylation. Non-PEGylated and PEGylated Pichia pastoris-produced rHuAChEs were administered intravenously to mice (n=3) at levels that were at least 30-fold higher than background. Residual AChE activity was determined in blood samples removed at various time periods.

CONCLUSIONS

We have shown that the large quantities of active AChE required for stoichiometric OP bioscavenging can be obtained, utilizing recombinant expression systems such as *P. pastoris*. The use of recombinant AChE also provides the means to incorporate favorable kinetic and pharmacokinetic characteristics into the enzyme fold by genetic engineering. Thus, by judicious elimination of specific PEG lysine target sites, we could generate well defined hypolysine versions of HuAChE (K23A/K348A/K470A/K496A trilysine AChE, and K23A/K332A/K348A tetralysine AChEs, containing 4 and 5 PEG target sites/enzyme subunit, respectively), which can be converted by PEGylation into uniformly modified enzyme forms displaying long-term circulatory residence and reduced immunogenicity, while exhibiting undiminished catalytic activity. The kinetic traits of the recombinant enzyme can be altered as well, so that the enzyme will be able to cope more efficiently with OP-mediated intoxication. For instance, replacement of phenylalanine[338] within the active center of the AChE molecule by alanine, resulted in a marked reduction of the rate of irreversible aging of the AChE-based bioscavenger. This

acquired property allows efficient *in-vivo* reactivation of the enzyme following exposure to nerve agents and thereby confers the enzyme with the ability to protect against repeated OP challenge. The concomitant introduction of such favorable pharmacokinetic and kinetic traits into a common enzyme mold, could provide the means to generate an optimal AChE-based bioscavenger for prophylaxis and treatment of OP-compound intoxications.

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